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REVERSIBLE RESPONSE TO PUROMYCIN AND SOME CHARACTERISTICS OF THE UPTAKE AND USE OF AMINO ACIDS BY UNFERTILIZED SEA URCHIN EGGS[1]

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Several groups have reported that all unfertilized sea urchin eggs incorporate labeled amino acids into protein, as judged by autoradiography (Bell and MacKintosh, 1967; MacKintosh and Bell, 1967; Epel, 1967; Tyler, Tyler and Piatigorsky, 1968). However, when incorporation is measured biochemically (*i.e.* by homogenization and acid precipitation of a radioactively labeled sample containing 1000 to 10,000 eggs) there is a strong theoretical possibility that a few "immature" eggs or ovarian fragments in the preparation could be contributing to it heavily. Thus experiments relying on measurement of the rate of incorporation in these preparations as a measure of the rate of protein synthesis in mature unfertilized eggs (MacKintosh and Bell, 1967; Epel, 1967) could be in substantial error, as suggested by Stavy and Gross (1967).

That it is not in error emerges from some basic facts presented in this work about the uptake and incorporation into protein of amino acids by unfertilized eggs. The basal level of incorporation in unfertilized egg preparations is, in fact, the rate of incorporation by mature unfertilized eggs, and is not attributable to contamination with immature cells. Support for this conclusion is based heavily on the differential response of unfertilized eggs as compared with oocytes to puromycin; the effect of the antibiotic on the former is reversible, while on the latter it is not. It is also based on other data which is presented below.

Materials and Methods

Animals and gametes

Specimens of *Strongylocentrotus purpuratus* were obtained from Pacific Bio-Marine, Venice, California. Specimens of *Arbacia punctulata* were obtained from Mr. Norris Hill, Beaufort, North Carolina. Animals were maintained at about 12° C in aerated tanks with subsand filters, and were used or discarded within two weeks after arrival.

Gametes were obtained by injection of isotonic (0.53 *M*) KCl, a method which yielded egg preparations that were superior (in terms of freedom from contaminating ovarian material) to those obtained by excision of gonads.

Eggs were prepared for use by passing them through four layers of cheesecloth, washing twice by settling in Millipore filtered sea water, and suspending them in

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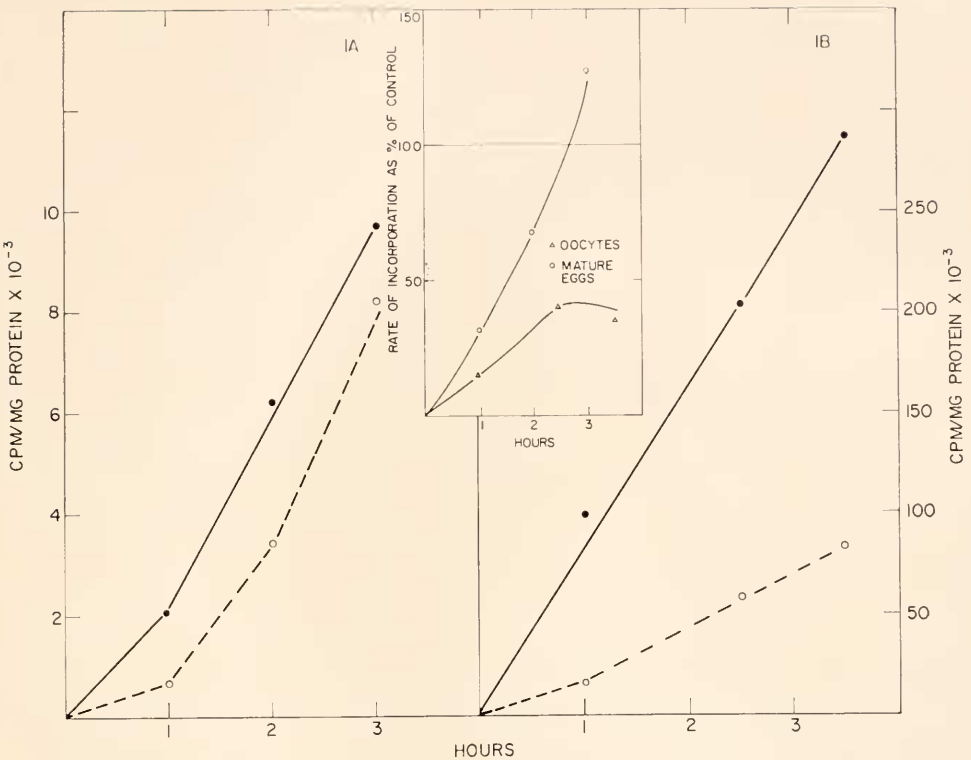

FIGURE 1. (a) Mature eggs of *Arbacia* were collected as described in Materials and Methods. Half were treated with puromycin at 235 μg/ml for four hours, while controls remained in untreated sea water. Both groups were rinsed and placed in fresh sea water, and maintained for an additional hour. ^{14}C amino acids were then added to both groups (1 μc/ml) and samples were withdrawn from both at hourly intervals for analyses as usual. Data are presented as acid insoluble cpm/mg protein for controls (●——●) and puromycin treated (○- - -○). Samples were counted at 11% efficiency. (b) An egg suspension containing 30% oocytes (eggs with a germinal vessel) was prepared by mincing the gonads of a female of *Arbacia* after it had been induced by KCl injection to shed most of its mature eggs. The preparation was divided in half and treated exactly as above, except that puromycin treatment was for only 1.5 hours, and radioactivity was counted at 90% efficiency. The data of Figure 1a and 1b is summarized in the inset, recalculated as rate of incorporation relative to controls, and plotted together for comparison of mature eggs (○——○) and oocytes (△——△).

Millipore filtered sea water containing penicillin (160 μg/ml) and streptomycin (100 μg/ml). Eggs were maintained at 20° C (*Strongylocentrotus*) or at room temperature (*Arbacia*), which varied from day to day between 21° and 24° C. in Erlenmeyer flasks on a rotary shaker at 60 to 70 rpm.

To determine their maturity and condition, we examined egg preparations microscopically before use, and discarded any with an excessive number of oocytes, fragments of ovaries or fertilized eggs. Oocytes are easily detected by the presence of a large germinal vessicle. Typical levels of contamination with oocytes were of the order of 0.1 to 0.3 per cent. Bacterial contamination was occasionally

monitored by plating samples of the egg suspension on agar made up in 80 per cent sea water and 20 per cent Charity Waymouth medium. Typical levels of contamination within five or six hours of the start of an experiment were of the order of 10–20 viable bacteria per ml. The maximum found was 1280 bacteria per ml, after over 24 hours of incubation of a culture. On the most gratuitous possible assumption a single bacterial cell could not yield more than 0.2 DPM (if uniformly labeled with ^{14}C at 50 mc/mMole). Thus in no case could contamination represent more than 3% of the radioactivity of a sample, and in most cases the amount would be far smaller than this. This level of contamination is not significant. Tyler, Tyler, and Piatigorsky (1968) also report negligible bacterial contamination.

TABLE I

Uptake of ^{14}C amino acids by eggs after short exposure and after rinsing

Exposure time	No rinse	1 rinse	4 rinse
3 min	—	3,900	—
3 min	—	4,050	—
1 hr	106,000	94,100	94,700
1 hr	102,000	97,000	95,600

Eggs of *Strongylocentrotus* were labeled for times indicated with 1 μc/ml ^{14}C amino acids. "No rinse" eggs were pipetted into 10 volumes of sea water (20° C) in a conical centrifuge tube, sedimented, resuspended and transferred to a clean tube to be dissolved in 8 *M* urea withuot further rinses. "1 rinse" consisted of suspending "no rinse" eggs in 2 ml cold homogenization medium and resedimenting and dissolving in 8 *M* urea. "4 rinses" consisted of three sea water washes prior to the wash with homogenization medium. Data are cpm/mg protein in the homogenates. Each figure represents a single determination on a single homogenate. All samples came from the same egg preparation.

Assay of incorporation of radioactivity, even at the maximum observed levels of contamination

^{14}C labeled reconstituted protein hydrolysate (a mixture of L amino acids with an average specific activity of about 140 mc/mMole, obtained from Schwarz Bioresearch, Orangeburg, New York) was used as a protein precursor. To measure incorporation in eggs which had been exposed to labeled precursor, a 0.5 or 1.0 ml sample of egg suspension was withdrawn from the incubation vessel and pipetted into a 12 ml conical centrifuge tube containing 10 ml of ice cold sea water. The eggs were sedimented by a brief centrifugation (about 5 seconds at full power in a clinical centrifuge) and the supernatant withdrawn. The eggs were then rinsed once with 2 ml cold homogenization medium [0.25 *M* sucrose, 0.24 *M* NH_4Cl, 0.01 *M* $MgCl_2$, 0.01 *M* Tris, pH 7.4; described by Spirin and Nemer (1965)] and dissolved overnight in 8 *M* urea. The urea treatment was found to render soluble the radioactivity which was hot acid labile, *i.e.*, such non protein incorporation as amino acyl t-RNA labeling. The urea homogenate was routinely analyzed as follows: a portion was precipitated by the addition of bovine serum albumin as carrier if needed and 30 per cent (w/w) TCA to give a final concentration of 15 per cent TCA and the precipitate collected on Millipore filters

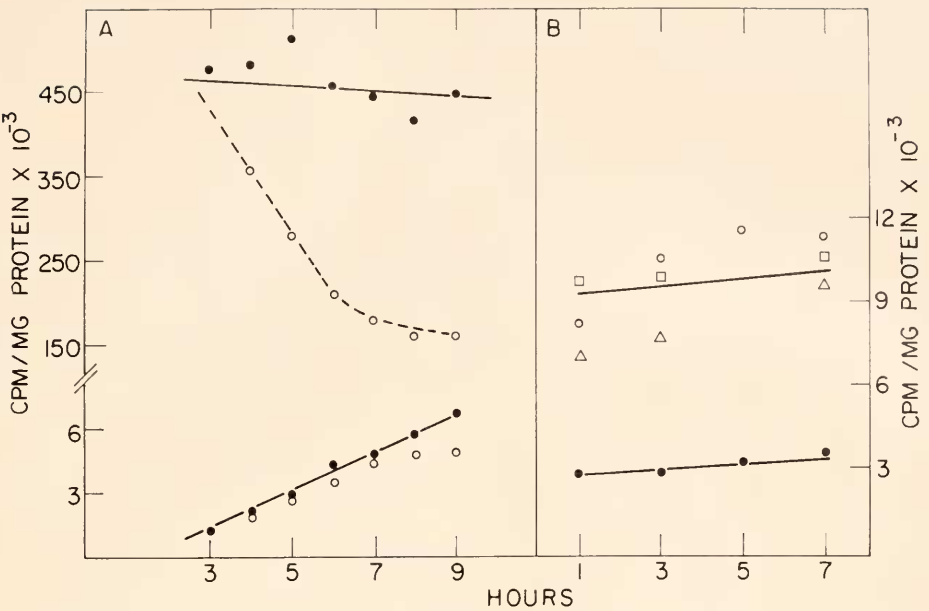

FIGURE 2. (a) Unfertilized eggs of *Strongylocentrotus* were exposed to 1 μc/ml ^{14}C amino acids for three hours, washed, and placed in sea water (●) or sea water containing 0.01 *M* ethionine (○). Both groups incorporated labeled amino acids from their endogenous pools into acid insoluble material (lower curves). The level of uptake (upper curves) remained essentially constant for controls, *i.e.*, the labeled amino acids accumulated during the three hour exposure do not wash off or leak out. Eggs exposed to ethionine lost a substantial portion of their endogenous label during this time. Note that ethionine does not significantly affect the rate of incorporation from the endogenous pool into protein until after four hours of treatment. (b) Eggs were maintained in sea water (●) or sea water containing unlabeled amino acids (methionine □, ethionine ○, or leucine △) at 0.01 M for five hours, washed, and given one hour pulse labels with ^{14}C amino acids at intervals thereafter. The eggs which were exposed to unlabeled amino acids incorporate at a rate 3.0 times higher than controls (overall average). The overall loss of amino acids from pools caused by the amino acid pretreatment, as judged from Figure 2a, is a factor of 2.8. Thus the effect of the amino acid pretreatment seems to be the exchange of the internal pools of many amino acids for the single amino acid present in the medium, lowering the size of pools of all amino acids except that supplied by approximately a factor of three and thereby inflating subsequent incorporation rates by a factor of three. That the value of cpm in the homogenate (uptake) accurately reflects these changes is evidence for its validity as a measure of radioactivity in endogenous amino acid pools.

in the usual way; these were counted with a low background gasflow counter at 11 per cent efficiency or in a Packard Tri-carb scintillation counter at 90 per cent efficiency; a second portion of the homogenate was used for estimation of proteins by the method of Lowry (Lowry, Rosebrough, Farr and Randall, 1951); a third portion of the homogenate was pipetted directly into a scintillation vial and counted in an appropriate medium [either Bray's fluid (Bray, 1960) or a mixture of four parts ethylene glycol monoethyl ether and six parts of the usual toluene based scintillation fluid]. The value obtained in this manner, normalized to amount of protein in the sample, is referred to as "uptake."

The value obtained by the Lowry reaction for protein content of the samples was used to normalize uptake and incorporation value to equal amounts of protein, and therefore presumably to equal numbers of eggs.

Results

Differential response of mature and immature eggs to puromycin

When mature unfertilized eggs are exposed to puromycin for four hours or less (at physiological temperatures), and then placed in puromycin-free water containing ^{14}C amino acids, within three hours they attain a rate of incorporation similar to that observed in untreated controls (Fig. 1). Slightly longer treatment with puromycin is followed by a stimulation of protein synthesis as reported previously (MacKintosh and Bell, 1967). Irreversibility begins to appear only after 6 hours

Table II

Incorporation of ^{14}C amino acids by eggs into acid soluble and insoluble material in the presence of unlabeled amino acid in the medium

	Uptake	Acid insoluble	Ratio
control	210,000	2133	0.0100
ethionine	24,000	219	0.0091

Eggs of *Strongylocentrotus* were exposed for one hour to 1 μc/ml ^{14}C amino acids in the presence or absence of 0.01 *M* ethionine. Samples were assayed as usual for radioactivity in the homogenate and for acid insoluble incorporation. Values are per mg protein.

of treatment of unfertilized eggs of *Arbacia*, and after more than 8 hours of treatment of unfertilized eggs of *Strongylocentrotus*.

On the other hand, when fertilized eggs are treated with puromycin, the effects become irreversible within one hour of treatment (Ellis, 1966). Similarly, when preparations of unfertilized eggs containing 30 per cent immature eggs (oocytes) are exposed to puromycin for 1.5 hours, protein synthesis in preparations washed free of the antibiotic is greatly depressed. After 3 hours in puromycin-free medium, incorporation in a population of mixed mature and immature eggs is reduced to 35 per cent of controls (Fig. 1b). The capacity of mature unfertilized eggs to recover from puromycin treatment is seen in Figure 1a. The rate of incorporation of precursor into acid insoluble material has exceeded that of controls by 3 hours after washing with puromycin-free sea water.

The effect of concentration and competition on uptake and use of radioactive amino acids

The value obtained by counting radioactivity in the total homogenate is believed to represent uptake of amino acids from the medium as opposed to some type of nonspecific absorption on the following basis:

(1) Eggs which are exposed to ^{14}C amino acids briefly have very low uptake values (Table I).

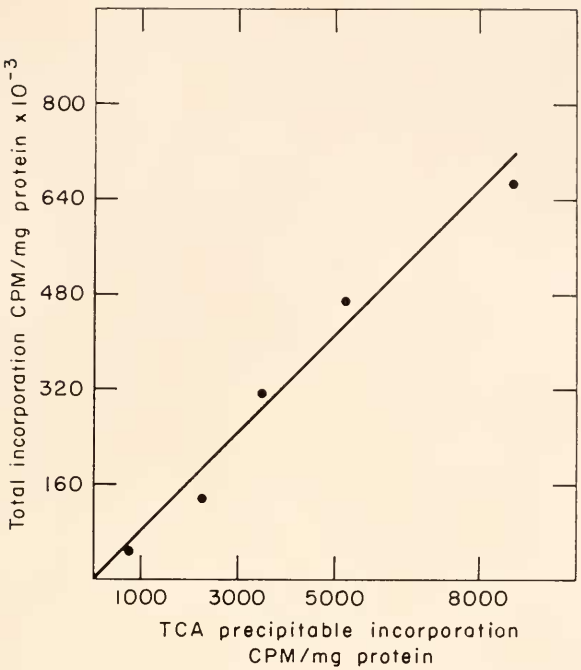

FIGURE 3. *Arbacia* eggs were pulse labeled for one hour with the following concentrations of ^{14}C amino acids: 0.1, 0.2, 1.0, 2.0, 5.0 μc/ml. Eggs were processed as usual and the acid insoluble radioactivity plotted against the total radioactivity in the homogenates. The linearity of the resulting curve shows that incorporation into acid insoluble material is a constant fraction of uptake independent of the actual level of uptake.

(2) Rinsing the eggs does not substantially change the value obtained (Table I).

(3) When eggs are labeled with ^{14}C amino acids, washed and returned to sea water, their uptake (counts in whole homogenate) does not change substantially for several hours (Fig. 2a). (This is also true of eggs exposed to puromycin after labeling.)

(4) If eggs are labeled and washed and placed in sea water containing a single nonradioactive amino acid at 0.01 *M*, their content of radioactivity (uptake) decreases threefold over a period of five hours. Conversely, if the eggs are first exposed to the unlabeled amino acid for five hours and then exposed to labeled amino acids, their rate of uptake is unchanged but their rate of incorporation into acid insoluble material is trebled. See legend, Figure 2b, for our interpretation of this effect. The effect of preincubation with amino acids was also observed by Tyler, Piatigorsky and Ozaki (1966).

(5) If eggs are exposed simultaneously to a single unlabeled amino acid and to the labeled mixture, their uptake is strikingly reduced, and incorporation into acid insoluble material is initially reduced in exact proportion to the reduction in uptake (Table II).

(6) If eggs are exposed to varying concentrations of ^{14}C amino acids uptake is not directly proportional to concentration, but incorporation into acid insoluble material is directly proportional to uptake (Fig. 3).

When unfertilized eggs of the two species were given one hour pulse labels with ^{14}C amino acids over an extended period of time, they displayed an essentially constant rate of protein synthesis (Fig. 4). The incorporation was not only constant on a per milligram protein basis, but was also a constant fraction of amino acid uptake. This constant rate of incorporation was also observed in eggs maintained continuously in ^{14}C amino acids.

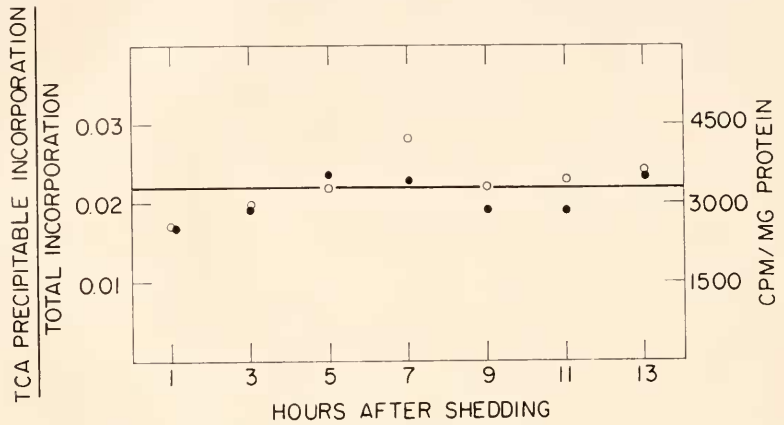

FIGURE 4. Unfertilized eggs of *Strongylocentrotus* were pulse labeled for one hour with 1 μc/ml ^{14}C amino acids at intervals over several hours. Results shown are cpm/mg protein (●) and fraction of uptake incorporated (○).

DISCUSSION

Two types of sea urchin cells which are known to be highly active in protein synthesis [those of embryos (Ellis, 1966) and oocytes (Fig. 1)] have been found to be irreversibly inhibited when returned to puromycin-free sea water after previous exposure to puromycin. In contrast, preparations of mature unfertilized eggs recover to normal rates of protein synthesis after puromycin treatments shorter than 4 hours and do not exhibit signs of irreversible inhibition for at least 6 hours. These observations imply that there is not a detectable fraction of the incorporation of ^{14}C amino acids in preparations of mature unfertilized eggs which can be attributed to either of these classes of highly active cells (oocytes and ovarian fragments, embryos). This finding supports the validity of previously published comparisons of the rate of incorporation in fertilized and unfertilized eggs (Epel, 1967; MacKintosh and Bell, 1967; Bell and MacKintosh, 1967).

A question raised by the foregoing results concerns the basis for the differential response to puromycin which remains to be elucidated.

The experiments on uptake of amino acids and its relation to incorporation of label into proteins establish that surface absorption or other nonspecific uptake is unlikely to be a major factor in this value. The uptake value provides what

appears to be a self-consistent measure of radioactivity in endogenous amino acid pools under a wide variety of circumstances. Thus the use of this value in verifying the absence of permeability changes (MacKintosh and Bell, 1967; MacKintosh and Bell, 1969) seems justified.

Summary

Protein synthesis in embryos and oocytes is irreversibly inhibited when the cells are returned to puromycin-free sea water after previous exposure to puromycin. In unfertilized eggs, on the other hand, it is not irreversibly inhibited. This effect and others are used to show that mature unfertilized eggs are actively engaged in protein synthesis, and that direct assessment of the relative rate of protein synthesis in mature eggs is not hindered by contamination with a small population of very active cells.

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